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CHANGES IN MICROTUBULES OF CILIA AND FLAGELLA FOLLOWING NEGATIVE STAINING WITH PHOSPHOTUNGSTIC ACID[1]

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Phosphotungstic acid (PTA) is a reagent commonly used for the negative staining of cilia, flagella and certain other structures (see, for example, Burton, 1970). In addition to its more or less passive role in outlining and filling such structures, however, there are also macerating and digestive effects on microtubules, and very little direct attention has been paid in the literature to these. The protein-precipitating action of PTA at pH 5 is well known, but to my knowledge its lysing action at pH 6.8 has never adequately been documented. The present report is an attempt to do so, and to give some data on the selective nature of such effects among various types of microtubules.

Materials and Methods

Both freshwater and marine turbellarian flatworms were used, the former collected locally from University Lake and from Stone Mountain, Georgia, and the latter furnished by the Supply Department of the Marine Biological Laboratory at Woods Hole, Massachusetts. They included the following: *Mesostoma georgianum, Microdalyellia* sp. and *Macrostomum* sp., all freshwater rhabdocoels; the alloecoel *Monoophorum* sp. (marine); and a marine polyclad, *Stylochus zebra. Prostoma rubrum,* a freshwater rhynchocoel, was also studied.

Maceration and negative staining were done with a 1% aqueous solution of phosphotungstic acid brought to a pH of 6.8 by the addition of 1 *N* KOH or NaOH. A trace of bovine serum albumin was added just before use. The larger animals were cut up with sharp needles and the pieces immediately put into PTA; smaller forms, such as *Macrostomum* and *Microdalyellia,* were dropped intact into the reagent. At appropriate intervals, samples were removed to Formvar-carbon-coated 200-mesh copper grids and treatment for all was continued at room temperature for periods varying from 2 to 10 min, after which the PTA was rapidly withdrawn with filter paper and the preparation allowed to dry. Micrographs were made with the Zeiss 9A and 9S electron microscopes.

Microtubules both of cilia (*Prostoma, Microdalyellia, Monoophorum, Mesostoma* and *Stylochus*) and of flagella (spermatozoa of *Mesostoma*) were studied, as well as the ciliary rootlets of *Macrostomum.*

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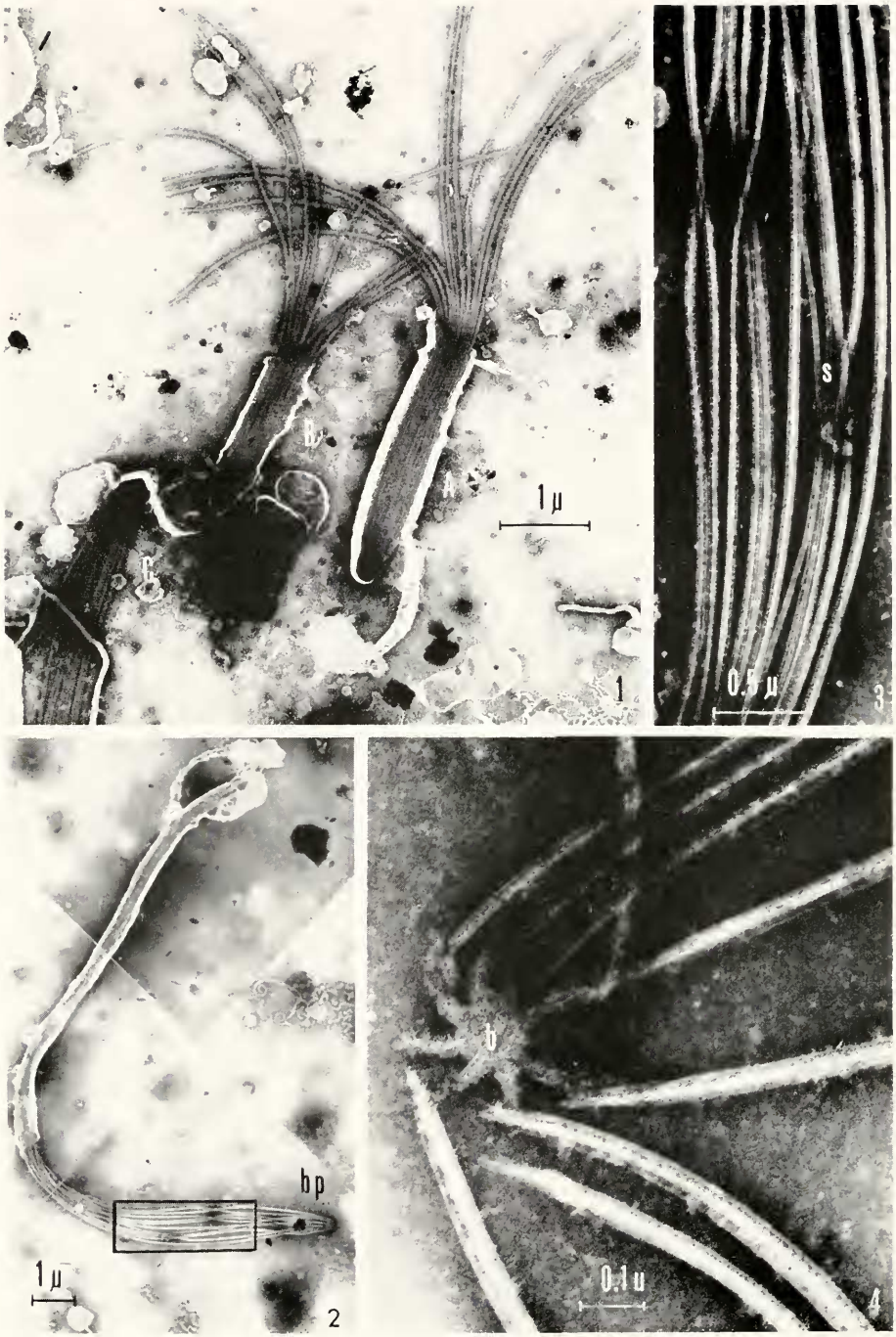

FIGURES 1–4.

Observations

Disappearance of one or both of the two central singlets in PTA-treated cilia and flagella, in the absence of noticeable effects on the doublets, is one of the very few predictable findings. This is well illustrated in Figure 1, showing 3 cilia, in varying stages of maceration but in close physical juxtaposition to one another on the same grid square. In cilium A (right) all 9 doublets and the 2 central singlets are present; the boundary of the binding matrix (arrow) is considerably farther distal along the axoneme than it is in cilium B, and the microtubules are longer. Furthermore, one of the 2 central singlets in B is entirely absent and the other is present for only a short distance. There is some evidence in higher-magnification micrographs that at least one of the doublets in B has likewise begun to disintegrate. In cilium C (lower left corner) the binding matrix has almost entirely disappeared and only the 9 doublets remain.

This variability in macerating action is also apparent among different grid squares of the same preparation, as shown by the cilia in Figures 2–5, all of which were on one grid. An entire cilium is shown in the montage of adjacent micrographs in Figure 2; here, very little maceration has occurred at the distal end of the cilium (upper), while at the basal region the binding matrix is absent and the 9 doublets and 2 singlets are well separated (Fig. 3), with very few, if any, signs of degeneration. The star-shaped basal plate in Figure 4 shows evidence of a considerable degree of degeneration; the 9 doublets are all present but most are broken off from the plate. The connections of the doublets to the basal plate appear to be effected by tapering processes.

The microtubules and basal plate of the cilium in Figure 5 are in a rather advanced stage of deterioration. Only portions of the 2 singlets (**s**) are still present and their protofibrils are conspicuous. There is considerable variation along the lengths of the 9 doublets. Both subtubules are mostly intact in the two doublets marked with white arrows; all the others have at least portions of one member absent. There appears to be a general tendency for the degradation process to begin proximally and proceed to completion distally, but sometimes this progression is interrupted, as at the breaks indicated by **b**. Behnke and Forer (1967) and Stephens (1970) have demonstrated that the B-member of the doublet is more thermolabile than the A-. Since the surviving members of

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Figure 1. Three cilia in varying stages of maceration, from the same grid square. In cilium A, all 9 doublets, the 2 central singlets, the matrix and membrane (the position of which is designated by the arrow) still are present. In cilium B, all 9 doublets are present but only 1 of the 2 singlets, and that for only a short distance. Nearly all the binding material has disappeared from cilium C, as well as both central singlets, leaving just the 9 doublets; *Prostoma rubrum;* 4-min treatment.

Figure 2. A montage of adjacent micrographs of a cilium; the basal plate end (**bp**) is well macerated and the 9 doublets and 2 central singlets are well defined here, but the membrane and matrix, and possibly some coagulated mucus, are still present along the distal two-thirds of the axoneme; *Microdalyellia;* 10-min treatment.

Figure 3. Enlargement of the area indicated on Figure 2. The two singlets lie on either side of the letter **s**. Scale designation should be 0.4 micron instead of 0.5.

Figure 4. End-on view of a ciliary basal plate (**b**); only the 9 doublets are present, and of these, only 4 have even a trace of remaining attachment to the plate; *Microdalyellia;* 10-min treatment.

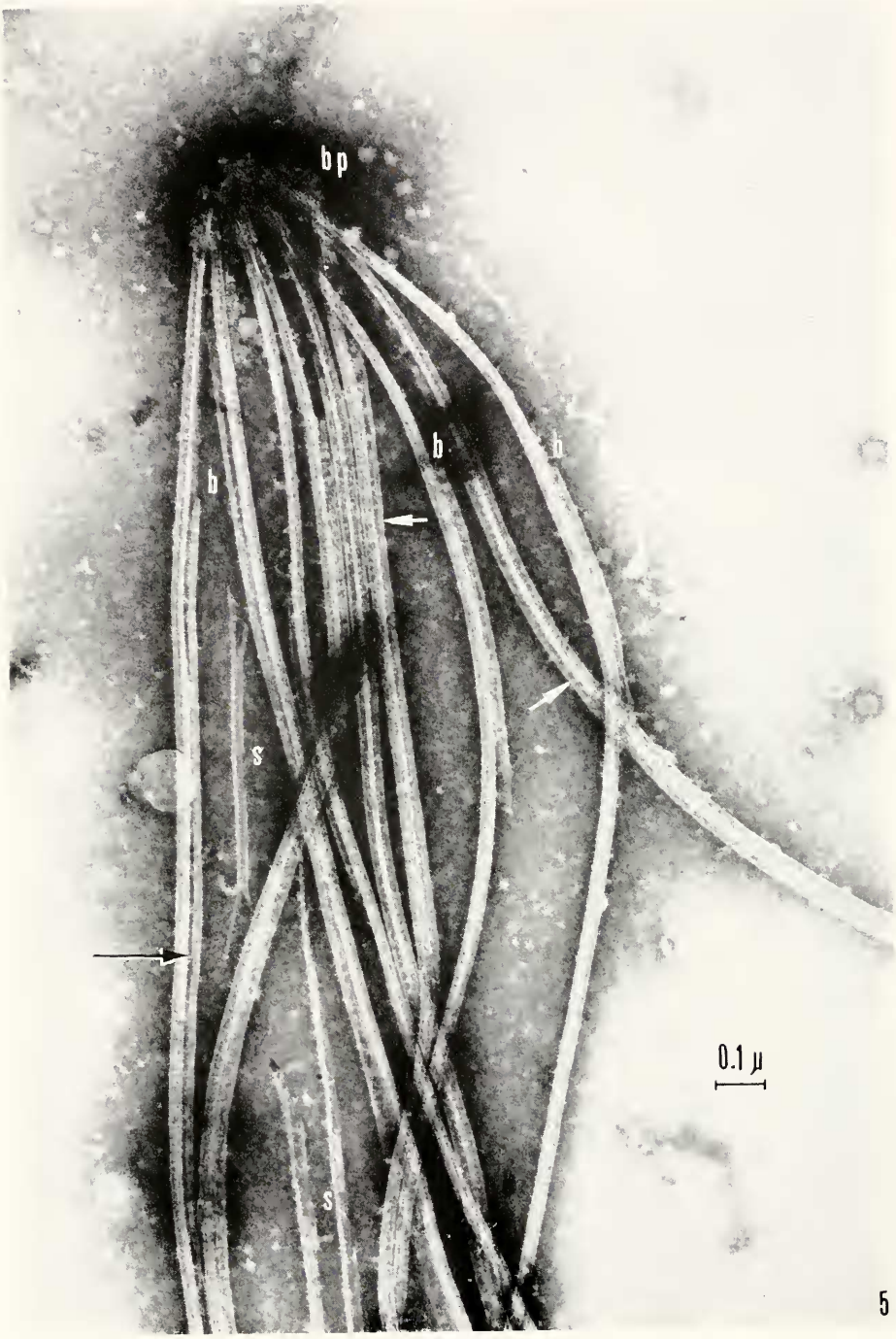


FIGURE 5.

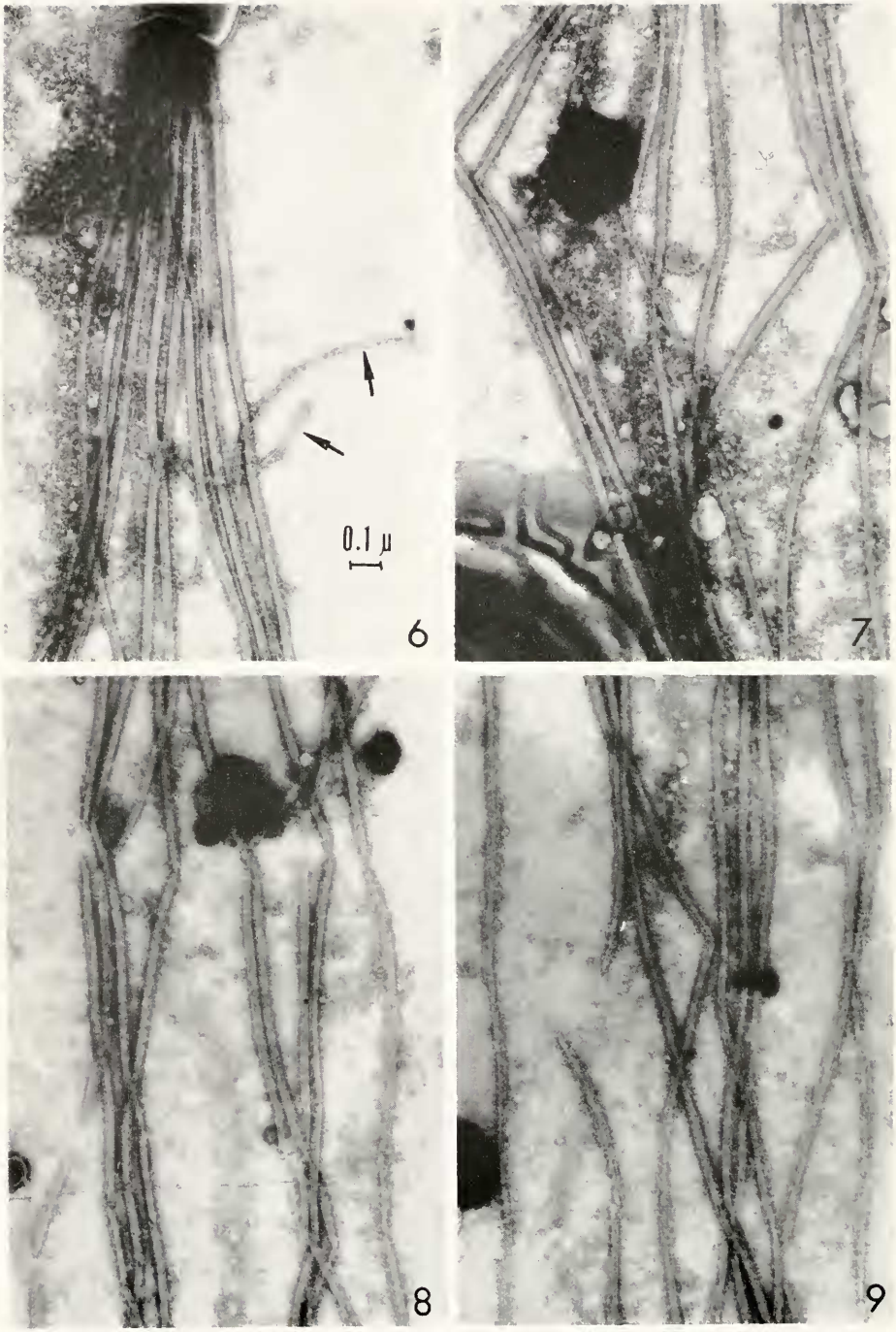
each doublet in Figure 5 have complete walls, and since the A-member is known to be the complete one (Stephens, 1970), it seems safe to surmise that we have another example of the greater sensitivity of the B-tubule to chemical and physical agents (in this case, PTA). Besides bringing about a complete disappearance of the B-subtubule, the macerating action of PTA may result in localized loss or separation of some of the component protofibrils for short distances (black arrow), so that a longitudinal split is present.

Figures 6–9 are micrographs of successive levels along a cilium *ca.* 14 microns long; Figures 6 and 7 are immediately adjacent to one another, as are Figures 8 and 9. Here the macerating action of PTA after a 10-min treatment differs in another way from the effects discussed above. The two central singlets (arrows in Figure 6) have not yet disappeared, but have a very frayed appearance, with conspicuous protofibrils. This is also the case for the doublets along their entire lengths, although it is less striking than in the singlets. There is little evidence in the doublets of the white line which, in optimally "stained" PTA-treated material, marks the common wall between the A- and B-subtubules of a doublet. Quite frequent breaks also occur along the doublets, as well as bends (Fig. 7).

An even more drastic macerating action of PTA is seen in the ciliary microtubules shown in Figure 10. Degradation here is so advanced that one cannot state with certainty which are doublet and which singlet microtubules; only 8 are present of the expected total of 11 and there was no evidence nearby on the grid square of the presence of the others. The existence of a helical arrangement of subunits is suggested at the point marked by the arrow, and elsewhere as well.

A difference in the effects of PTA on flagellar axonemes and on the cortical singlets of the spermatozoon of *Mesostoma* (Henley, Costello, Thomas and Newton, 1969) is shown in Figure 11. Here, after a 3-min treatment, the flagellar axoneme (**fa**) is still almost unaffected by PTA, while the cortical singlets (**cs**) are thrown into a striking helical configuration. It is not apparent in this low power micrograph, but in addition to the gross PTA-stimulated spiralling, the cortical singlets have a marked helical pattern in their walls, when viewed at higher magnifications. This is very similar to the configuration demonstrated in cortical singlets of the spermatozoon of *Stylochus* by Thomas (1970). We have thus far found no evidence of comparable helical configurations in cilia on the same grids as the coiled cortical singlets of spermatozoa, suggesting that here is another differential effect of PTA on different types of microtubules. The spiralling of the entire complement of cortical singlets shown here can readily be related to an abrupt coiling of certain spermatozoa (including that of *Mesostoma*) observed by phase contrast microscopy during treatment of living spermatozoa with PTA. This coiling may persist for a period of 20 min or more of continued treatment, after which it disappears and the spermatozoa again assume a more straightened form. There is thus the possibility that at a certain stage of its action, PTA stimulates the microtubule subunits to undergo some of the changes normally associated

FIGURE 5. Considerable maceration of the microtubules has occurred in this cilium. The 9 doublets and fragments of the singlets (**s**) are present, but only the 2 doublets designated with white arrows are intact or nearly so. One member of the doublets (probably the B-subtubule) has completely disappeared at the regions marked **b**, and a longitudinal separation between the two is apparent at the black arrow, **bp**, basal plate; *Microdalyellia;* 10-min treatment.



FIGURES 6–9.

with the production of motility. Continued action of the PTA macerates the microtubules more, and their "contractility" is lost. There is as yet no way of knowing whether PTA induces spiralling of the singlets, or whether the coiling results from release of an inherent tendency towards spiralling which is facilitated by PTA's dissolution of the spermatozoon's plasma membrane.

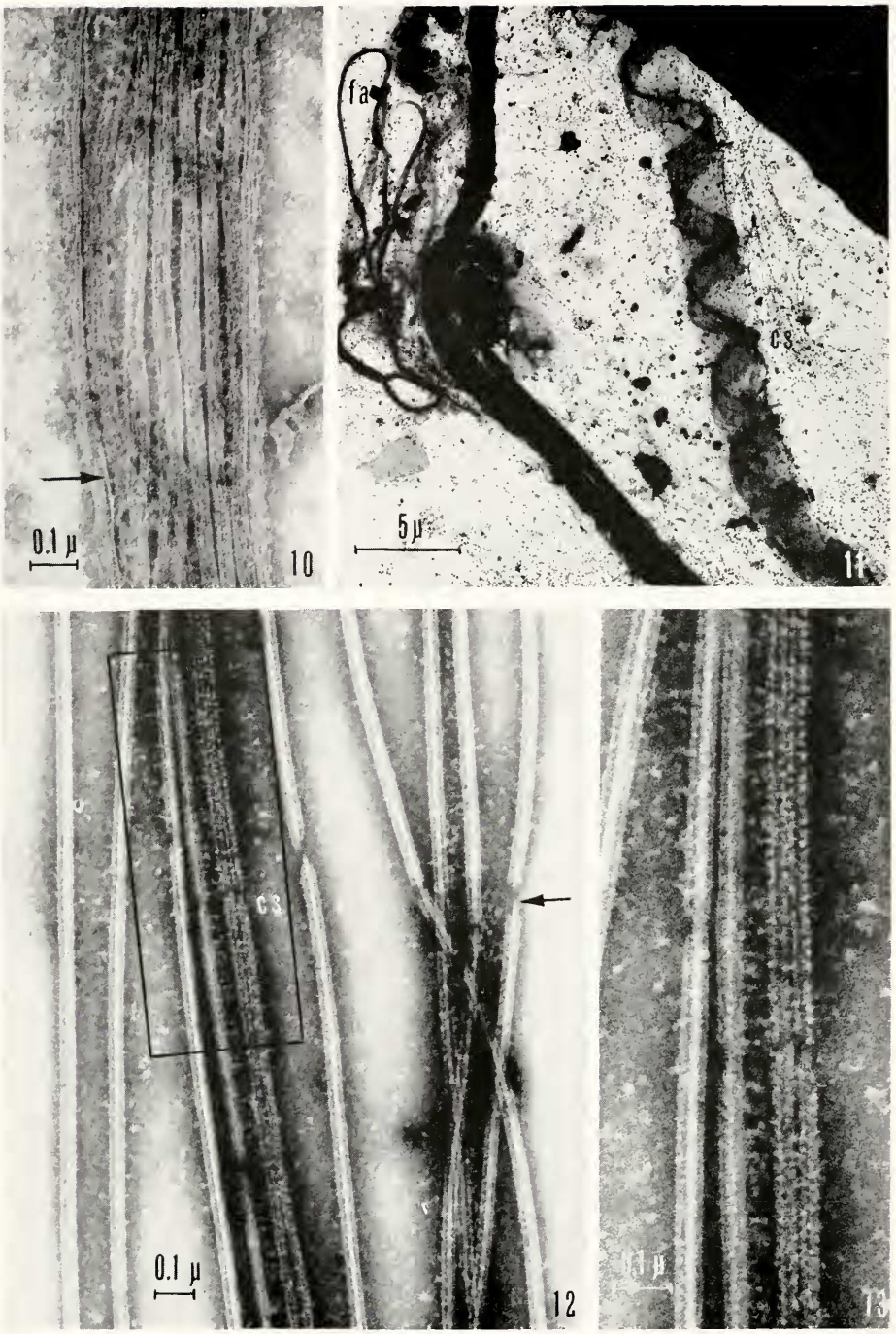
The pH of the PTA solution appears to be of rather critical importance, for even very short treatments at pH 8.3 (Figs. 12 and 13) result in changes in the 2 central singlets of cilia which are quite unlike those observed after any other treatment thus far tested. The singlets become semi-fused together along much of their lengths, and their component protofibrils have a beaded appearance, similar to that described by Behnke and Forer (1967) for central singlets after negative staining at pH 7. Some breaks are also apparent in the doublets, but both members reappear beyond the breaks. The common walls between the subtubules can be seen to persist in the absence of members of the doublet at the point marked by an arrow.

One or more striated ciliary rootlets per cilium are present in sections of all the forms studied here, but are rarely encountered in PTA-treated material. This suggests that they are very sensitive to the digesting action of the reagent at the concentration and pH used, and that they therefore disappear rapidly. Figure 14 shows two rootlets which did survive the treatment; there is a conspicuous major periodicity of *ca.* 790 Å and evidence of a longitudinal fibrous substructure as well. The montage of adjacent micrographs in Figure 15 shows a rootlet in which the process of degeneration is quite far advanced at one end, while the other end (at the top of the picture) still retains the clear 790 Å repeating pattern. The longitudinal fibrous substructure is clear in the lower region, but the periodicity here is obscured. Dorey (1965) observed a 650–700 Å repeating pattern in negatively stained ciliary rootlets from a number of acoels; he used 0.05% PTA at pH 6.2 (as opposed to 1% at pH 6.8 used in the present work) and treated for periods of 5–10 min. The differences in technique may account for the disparity in periodicity, or there may be species differences. Further work to elucidate this point is in progress.

Discussion

The impressive variety of degenerative changes in microtubules and associated structures, and the general unpredictability of effects exerted by PTA lead one to the belief that perhaps a great deal of caution should be exercised in the interpretation of electron micrographs of negatively stained material. It is not clear, for example, exactly which of the changes described by such workers as Behnke and Forer (1967) and Burton (1968) were due to experimental manipulations (using such agents as colchicine, temperature, pepsin, *etc.*) and which to the action of PTA itself.

Figures 6–9. Micrographs at successive levels along one cilium, showing conspicuous protofibrils and many breaks. The complete series of 7 micrographs comprising this group reveals that every doublet has at least one break along its length, and several have more than one. The arrows in Figure 6 designate the disintegrating central singlets. Note the rather sharp bends in the microtubules of Figures 7 and 9. Figures 6 and 7 are immediately adjacent to one another, as are Figures 8 and 9; *Monoophorum;* 10-min treatment.



FIGURES 10-13.

Behnke and Forer (1967) found that the central singlets of the 9 + 2 configuration were more likely to be absent in PTA-treated material, confirming the results reported here and by many other workers (see their Table 3). Grimstone and Klug (1966) showed that the central singlets in flagella of a number of protozoa from *Cryptocercus* and *Zootermopsis* only rarely survived even very short treatments (15 sec) with 1% PTA, pH 7.0. If the central singlets were present, however, these were observed to be uncollapsed, in contrast to the doublets which readily collapsed or frayed.

Another type of central element in axonemes has been studied by Burton (1968) and Thomas (1970), among others, in spermatozoa of the lungfluke *Haematoloechus* and the polyclad turbellarian *Stylochus*, respectively. They both found the complex central core of the "9 + 1" pattern to be more resistant than either the doublets or the cortical singlets to a variety of experimental treatments, followed by negative staining with PTA. These data of Burton and of Thomas for spermatozoa are in interesting contrast to those presented here for cilia, which implicate the central elements as the least resistant to the action of PTA. However, the relationship of central singlets in the 9 + 2 pattern to the comparatively massive core in the "9 + 1" pattern is obscure and probably complex. It may be, also, that there is an inherent difference in the properties of microtubules in cilia and in spermatozoa of the same form. Paired "9 + 1" axonemes, as well as cortical singlets, are found in spermatozoa of 3 (*Mesostoma*, *Microdalyellia* and *Stylochus*) of the 6 forms included in the present report. The spermatozoon of *Macrostomum* has cortical singlets only, with no axonemes (Henley, unpublished data). In the other two (*Monoophorum* and *Prostoma*) the axonemal pattern of spermatozoa has not yet been studied, but on the basis of information we have gathered from 8 other free-living flatworms, 1 other rhynchocoel and 3 annelids, it is possible to state that there is no set relationship between the pattern of microtubules in spermatozoa and in cilia of the same form. In addition, the fact that the cortical singlets of the spermatozoon of *Mesostoma* (and of *Microdalyellia* and *Stylochus* as well) react differently to PTA than do the microtubules of the flagella suggests yet another source of variability.

Differences in susceptibility of microtubules to PTA (and to other treatments as well) such as those discussed above may very well involve subtle differences in the biochemical composition of these structures in the various groups, despite their

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FIGURE 10. Ciliary microtubules so completely macerated that one cannot determine which (if any) are singlets and which doublets. Arrow designates helical particulate substructure. Three of the normal total complement of microtubules are present; *Mesostoma;* 6-min treatment.

FIGURE 11. Cortical singlet microtubules (**cs**) of a spermatozoon (at right) are thrown into a conspicuous helical arrangement by PTA treatment, while the microtubules of the flagellar axoneme (**fa**) are not. The thick solid black structure in the center is probably an unmacerated spermatozoon; *Mesostoma;* 4-min treatment.

FIGURE 12. This ciliary axoneme was treated with PTA at pH 8.3, rather than the usual 6.8. All 9 doublets are present, as well as the 2 central singlets (**cs**), which appear to be almost fused together and which have a striking beaded appearance. The breaks in the doublets are somewhat sharper than those usually seen after treatment at the lower pH; *Stylochus;* 2-min treatment.

FIGURE 13. Enlargement of the area indicated by the rectangle in Figure 12. (Micrograph by Mary Beth Thomas).

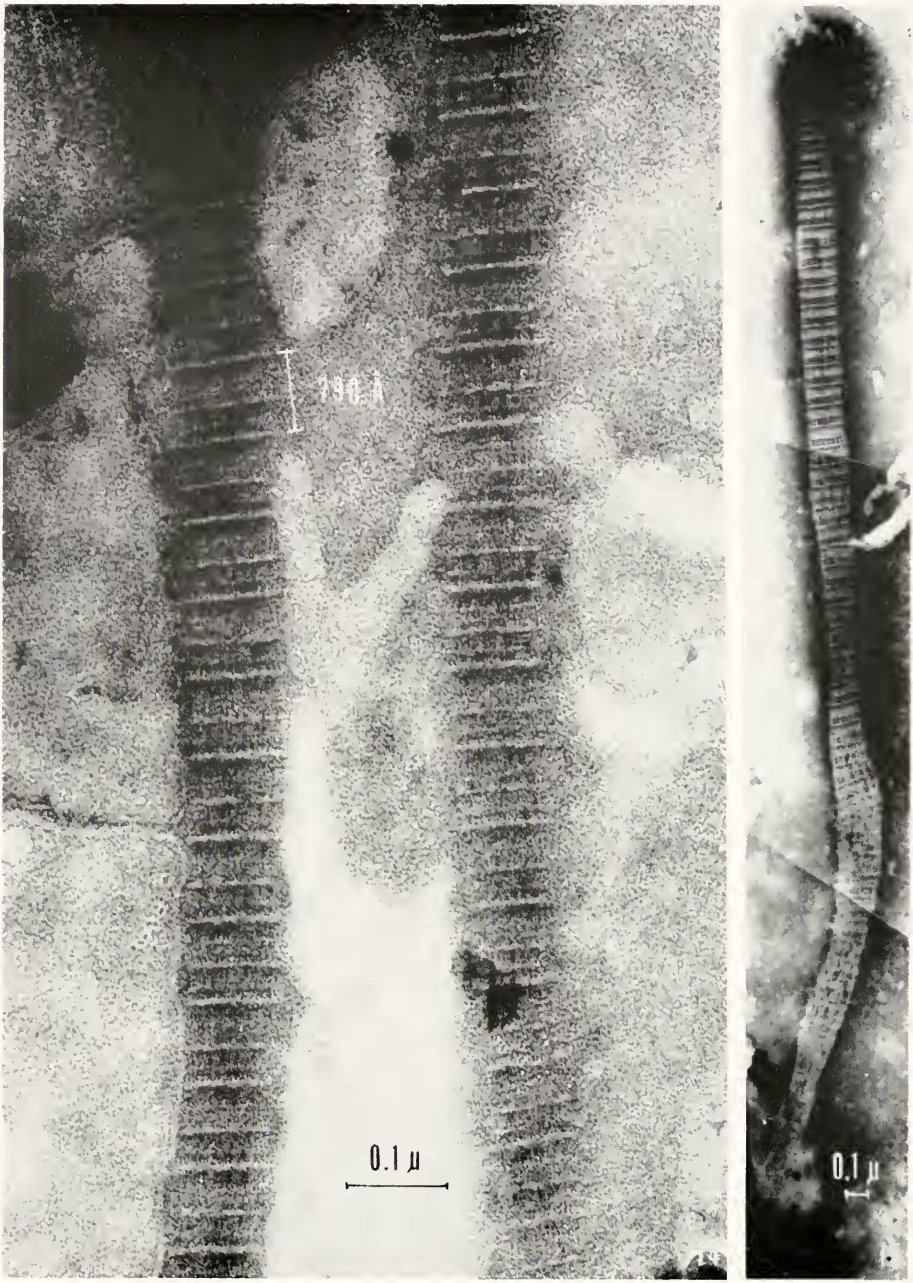

FIGURE 14. Two ciliary rootlets, negatively stained with PTA, showing a major repeating pattern of *ca.* 790 Å. Note the longitudinal fibrous substructure; *Macrostomum;* 4-min treatment.

apparent morphological resemblances. Evidence for this is offered by the recent work of Behnke (1970), who studied the comparative sensitivity of microtubules in disk-shaped blood cells of the frog, chick, rat and man, to cold, Colcemid, *N*-ethylmaleimide and alkaloids of *Vinca*. He found very marked species differences in such susceptibility. In our material, there are undoubtedly great variations in such features as the toughness of the pellicle, the presence or absence of mucus, *etc.*, which could affect the action of PTA and the required duration of treatment for adequate negative staining. Also, it is our experience that marine forms are considerably less sensitive to the macerating action of PTA than freshwater ones, perhaps because of the presence in seawater of divalent ions, which appear to inhibit the action of PTA. In this connection, Roth and Shigenaka (1970) have recently shown that microtubules of the heliozoan axopod respond to treatment with cupric ion very much as they do to colchicine. They were also found to be very susceptible to degradation in the presence of nickel ion. The concentrations of both these cations in sea water are low (copper, 0.001–0.09 mg/l, nickel, 0.0001 mg/l, according to the *Handbook of Chemistry and Physics,* 1970, page F-145), but they could well be involved in the observed variability in the action of PTA.

It may be pertinent to point out here that while the precise formula of phosphotungstic acid is not entirely clear (due to the amount of included water of crystallization), its molecular weight is high. Values of 3312.5 and 3132.4 are given (*Handbook of Chemistry and Physics,* 1970), for molecules containing 24 and 14 units of water, respectively. Therefore, a 1% solution is only about 0.003 *M*. Since the spermatozoa of many marine forms are osmotically adjusted to survive for a considerable period of time in sea water of molarity equal to that of a 0.55 *M* NaCl solution, the 1% PTA is decidedly hypotonic. Some of the gross changes in living sperm subjected to PTA treatment may be due to this factor.

One of the most unpredictable aspects of negative staining is the correct duration of treatment with PTA, to achieve optimal "staining" of the microtubules without overmaceration. This seems to be correlated, to some extent at least, with the species differences already alluded to; for each new form we study, the correct duration of treatment is determined empirically and has been found to range from a few seconds to 30 min or more.

Burton (1966) briefly discusses the variability in action of PTA, in material on consecutive grids treated in the "same" way, and points out (page 404) that studying "negatively stained material forces one to make value judgments." These value judgments involve such factors as taking into account the varying degrees of susceptibility of the different types of microtubules and other components of cilia and flagella, to the degenerative effects of PTA. That this is indeed the case is abundantly borne out by the results reported here, in negatively stained microtubules on the *same* grid and, in fact, on the same grid square.

FIGURE 15. Montage of three adjacent micrographs, showing progressive maceration of a negatively stained ciliary rootlet. The basal body end (top) is intact, but there is progressive disintegration, ending in a completely disorganized mass at the distalmost end; *Macrosotomum;* 10-min treatment.

I am indebted to Miss Mary Beth Thomas for permission to use her unpublished micrographs (Figs. 12 and 13), and to Miss Thomas and Dr. Donald P. Costello for valuable assistance in preparation of the illustrations.

Summary

1. Variability in the macerating action of 1% aqueous phosphotungstic acid, pH 6.8, is exemplified in microtubules of flatworm and rhynchocoel cilia, from one grid square to another of the same preparation and within a single grid square. The central singlets appear to be the most susceptible and are often completely absent, even in cases where the binding matrix is still present around the doublets. Maceration usually, but not always, begins at the distal tip of a cilium and proceeds towards the basal plate; it is evident along the lengths of doublets as partial or complete loss of one subtubule, as breaks and bends, and as fraying into the component protofibrils, with disappearance of the white line marking the wall between the subtubules.

2. Cortical singlet microtubules of a spermatozoon were thrown into a helical configuration by the action of PTA, while the flagellar microtubules of the same spermatozoon were unaffected.

3. After treatment with 1% PTA at pH 8.3, the central singlets of cilia (but not the doublets) were semi-fused along most of their lengths, and had a beaded appearance; there were breaks in the doublets but otherwise they appeared to be unaffected.

4. Ciliary rootlets were rarely seen, but when present had a clear 790 Å major repeating pattern and a longitudinal fibrous substructure.

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